Caspase 3 Activity and Lipoperoxidative Status in Raw Semen Predict the Outcome of Cryopreservation of Stallion Spermatozoa[1]

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ABSTRACT

Stallion-to-stallion variability in the quality of cryopreserved ejaculates postthaw affects the commercial acceptability of frozen semen and thus is a major constraint for the equine industry. In recent years, the molecular mechanisms associated with sperm damage during cryopreservation have become better understood. Identification of the freezability of the ejaculates before the freezing process is initiated will have a major impact on the equine industry. We studied three markers of oxidative stress in sperm, including 8-iso-PGF2alpha, 8-OH guanosine, and 4-hydroxynonenal (4-HNE); the presence of active caspase 3; and their changes after sperm cryopreservation. Although 4-HNE levels increased after cryopreservation (from 7% to 33%, $P < 0.001$), 8OH-guanosine and 8-ISO-PGF2alpha levels decreased after cryopreservation (from 130 to 35 arbitrary fluorescence units, $P < 0.01$, and from 1280 to 1233, $P < 0.01$, respectively). Postthaw sperm quality was classified as poor, average, or good using the 25th and 75th percentiles of all assays of sperm quality studied (motility, velocity, membrane functionality, and thiol content) as thresholds. Using these values, a sperm postthaw quality index was proposed. Receiver operating characteristic curves and the Youden J statistic were used to investigate the value of the measured parameters in fresh sperm as predictors of potential freezability. Using these techniques, we identified markers of bad freezers (percentages of caspase 3-positive dead sperm [area under the curve (AUC) = 0.820, $P < 0.05$] and percentages of caspase 3- and 4-HNE-positive sperm [AUC = 0.872, $P < 0.05$]) and good freezers (percentages of caspase 3-negative live sperm [AUC = 0.815, $P < 0.05$], percentages of live sperm with high thiol content [AUC = 0.907, $P < 0.01$], and percentages of 8-ISO-PGF2alpha-positive sperm [AUC = 0.900, $P < 0.01$]. Moreover, we described for the first time the presence of 8-ISO-PGF2alpha in stallion spermatozoa and revealed the importance of considering different markers of oxidative stress.

4-HNE, 8-ISO-PGF$_{2\alpha}$, 8-OH guanosine, cryopreservation, equids, flow cytometry, ROS, sperm, stallion

INTRODUCTION

International commerce of frozen semen is a major component of the equine industry [1]. Despite its great advantage in the equine male gamete trade, cryopreservation still has numerous drawbacks. The reduced lifespan of frozen-thawed sperm and the high stallion-to-stallion variability [2] in sperm quality postthaw are the two major limitations constraining the wider use of this reproductive technology [3]. Frozen-thawed stallion sperm undergoes accelerated senescence and a reduced lifespan in the mare reproductive tract, requiring artificial insemination (AI) to be conducted very close to the time of ovulation in order to obtain acceptable fertility [3]. This method requires intensive management of the mares, which increases the costs related to AI in horses and limits its use to specialized veterinarians in specific institutions. In recent years, the molecular mechanisms associated with this accelerated sperm senescence, or spermptosis [4], as it has recently been termed, have begun to be understood. Osmotic shock during cryopreservation [5] damages the mitochondria [6], leading to increased reactive oxygen species (ROS) generation. Unbalanced ROS leads to the production of toxic aldehyde adducts, such as 4-hydroxynonenal (4-HNE) [7], causing lipid peroxidation and dephosphorylation of Akt, which trigger the activation of caspase 3 and accelerate sperm senescence and death [8]. However, paradoxical results have been reported in terms of the impact of ROS on stallion sperm function. Beside the detrimental actions of lipid peroxidation and particularly of 4-HNE on sperm function, more robust and fertile spermatozoa are also high producers of ROS [9, 10]. This observation, in combination with an especially active mitochondrial metabolism in this specific sperm subpopulation, strengthens the concept of ROS homeostasis. Thus, with regard to ROS analysis, identification of toxic adducts is pivotal in diagnosing ROS toxicity separately from increased superoxide ($O_2^{\bullet-}$) leakage due to very active oxidative phosphorylation [11–13]. Factors modulating this dual response include the total thiol content in the spermatozoa, which is linked to survival [7], and the presence of dead sperm [14] in the media, which may accelerate senescence because of increased ROS. If these factors are in equilibrium, then the sperm will have a normal lifespan; if equilibrium is lost, spermptosis is triggered, and accelerated sperm senescence occurs [15].

Identification of stallions or ejaculates more suitable for freezing will expand the use of cryopreserved stallion sperm by preventing the marketing of low-quality products. However, a method to identify good ejaculates before freezing is still elusive. Nevertheless, reports have identified shape factors [16, 17] and the chemical composition of the plasma membrane [18] as suitable markers of freezability. Other reports have revealed the pivotal importance of the sperm mitochondria as a major source of energy in equine sperm [9, 11] and the importance of mitochondrial diagnosis in the identification of more fertile spermatozoa [9, 10]. Mitochondria play a pivotal role in the triggering of caspase 3 activation in spermatozoa [7, 19]. Based on this recent knowledge, we investigated mitochondrial, apoptotic, and antioxidant status according to different markers in fresh stallion spermatozoa using a wide variety of flow cytometry-based assays. We hypothesized that mitochondrial and apoptotic markers, along with the differences in the intrinsic antioxidant status of the spermatozoa, would enable the identification of samples with better cryoresistance. Using these parameters, we aimed to develop mathematical methods to forecast the quality of the samples after thawing.

MATERIALS AND METHODS

Reagents and Media

Hoechst 33342 (excitation [Ex] 350 nm, emission [Em] 461 nm; Ref: H3570), CellEvent Caspase 3 and 7 (Ex 502 nm, Em 530 nm; Ref: C10423), ethidium homodimer (Ex 528 nm, Em 617 nm, Ref: E1169), JC-1 (Ex 514 nm, Em 529 monomer form, 590 aggregate form; Ref: T3168), ThiolTracker Violet (Ex 404 nm, Em 526 nm; Ref: T10095), and the LIVE/DEAD Fixable Aqua Dead Cell Stain kit were purchased from Molecular Probes (Ex 405 nm, Em 526 nm; Ref: L34957). Anti-4-HNE antibody (HNEJ-2; Ref: ab48506), anti-8-iso-prostglandin F2α antibody (Ref: ab2280), goat anti-mouse IgG H&L (Alexa Fluor 647; Ex 652 nm, Em 668 nm; Ref: ab150115), and goat anti-rabbit IgG H&L antibody (Alexa Fluor 405; Ex 405 nm, Em 488 nm) were purchased from Abcam. The OxyDNA Assay Kit (Ref 500095 Ex 495 Em 515) was purchased from Calbiochem.

Semen Collection and Processing

The semen was obtained from eight fertile stallions (three ejaculates from each stallion), all of which had records of the successful insemination of mares, were regularly used as semen donors during the 2015 breeding season (February–June), and were maintained according to institutional and European regulations (Law 6/2913 June 11th and European Directive 2010/63/EU). The ejaculates were collected using a prewarmed (42°C) and lubricated Missouri model artificial vagina, with a filter to separate the gel fraction. The semen samples were immediately transported to the laboratory for evaluation and processing; because the collection barn is adjacent to the laboratory, less than 1 min was required to transport the semen. The ejaculate was separated into two aliquots, diluted 1:2 in INRA 96 (IMV), and centrifuged at $600 \times g$ for 10 min at room temperature. One of the aliquots was further diluted in INRA 96 to obtain a concentration of 100×10^6 spermatozoa/ml and was kept at room temperature (22°C) for analysis as fresh sample controls. The other aliquot was diluted in the freezing medium Cáceres (University of Extremadura, Cáceres, Spain) containing 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100×10^6 spermatozoa/ml. After loading the extended semen into 0.5-ml straws (IMV), the straws were ultrasonically sealed with UltraSeal 21 (Minitube of America MOFA) and immediately placed in an IceCube 14S (SY-LAB) programmable freezer. The following freezing curve was used [7]. Straws were kept for 20 min at 22°C, and they were then slowly cooled from 22°C to 5°C at a cooling rate of 0.5°C/min and kept at 5°C for 10 min. Thereafter, the freezing rate was increased to −30°C/min from 5°C to −140°C. The straws were then plunged into liquid nitrogen and stored until analysis. For the analysis, three straws per stallion and freezing operation were thawed in a water bath at 37°C for at least 30 sec and diluted in prewarmed INRA 96 extender to a final concentration of 50×10^6 spermatozoa/ml. All analyses were conducted immediately after thawing, except for the DNA oxidation analysis, in which a second evaluation was performed after 3 h of incubation at 37°C.

Computer Analysis of Sperm Motility and Velocity

Sperm motility and kinematic parameters were assessed using a computer-assisted sperm analysis system (ISAS Proiser). Semen was loaded in a Leja chamber with a 20-μm depth (Leja) and placed on a warmed stage at 38°C. The analysis was based on the evaluation of 60 consecutive digitalized images obtained using a 10× negative phase-contrast objective (Olympus CX41). At least three different fields were recorded to ensure that at least 200 spermatozoa were analyzed per sample. Spermatozoa with an average velocity (VAP) <15 μm/sec were considered immotile, whereas spermatozoa with a VAP >35 μm/sec were considered motile. Spermatozoa deviating <45% from a straight line were characterized as linearly motile. Three ejaculates per stallion (fresh and frozen-thawed samples) were evaluated.

Flow Cytometry

Flow cytometry analyses were conducted using a MACSQuant Analyser 10 (Miltenyi Biotech) flow cytometer equipped with three lasers emitting at 405, 488, and 635 nm and 10 photomultiplier tubes (PMTs): V1 (Ex 405 nm, Em 450/50 nm), V2 (Ex 405 nm, Em 525/50 nm), B1 (Ex 488 nm, Em 525/50 nm), B2 (Ex 488 nm, Em 585/40 nm), B3 (Ex 488 nm, Em 655–730 nm [655 long pass (LP) + split 730]), B4 (Ex 499 nm, Em 750 LP), R1 (Ex 635 nm, Em 655–730 nm [655LP + split 730]), and R2 (Ex 635 nm, Em filter 750 LP). The system was controlled using MACS Quantify software. The quadrants or regions used to quantify the frequency of each divided sperm subpopulation depended on the particular assay. Forward and sideways light scatter were recorded for a total of 50 000 events per sample. Nonsperm events were eliminated by gating the sperm population after Hoechst 33342 staining or MitoTracker Deep Red staining (Life Technologies). The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. The data were analyzed using Flowjo V 10 software. Unstained, single-stained, isotype, and fluorescence minus one (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest.

Detection of 8-ISO-PGF$_{2\alpha}$

The following stock solutions were prepared: LIVE/DEAD Fixable Aqua Dead Cell Stain kit (50 μl of dimethyl sulfoxide [DMSO] in the LIVE/DEAD vial) and MitoTracker Deep Red (500 μM in DMSO). The samples (1 ml) containing $6–7 \times 10^6$ spermatozoa/ml in PBS were stained with 1 μl of LIVE/DEAD Fixable Aqua Dead Cell Solution and 0.3 μl of MitoTracker Deep Red. After thorough mixing, the samples were incubated at room temperature (22°C) for 30 min in the dark. Then, the spermatozoa were washed with PBS and fixed with 900 μl of 2% paraformaldehyde in PBS. The samples were stored at 4°C, and the analysis continued the next day. Then, the spermatozoa were washed in PBS and incubated with 1 μl of anti-8-iso-prostglandin F2α antibody for 30 min at room temperature (22°C) in the dark after thorough mixing. Then, the spermatozoa were washed in PBS and stained with 4 μl of goat anti-rabbit IgG H&L antibody (Alexa Fluor 405), mixed, and incubated at room temperature (22°C) for 30 min in the dark. Finally, the samples were washed in PBS before reading in the flow cytometer.

Determination of DNA Oxidation Using the 8-Oxoguanine Assay

This assay is based on the direct binding of a fluorescent probe to the DNA adduct 8-oxoguanine (OxyDNA Assay Kit; Calbiochem), a major oxidation product and an important indicator of free radical-induced DNA damage and oxidative stress. The assay was performed according to the manufacturer's instructions and following previously published protocols for stallion spermatozoa [20–22]. Briefly, aliquots (5×10^6/ml) were obtained from each sample, washed in PBS, and fixed in a 2% paraformaldehyde solution in PBS (pH 7.6) for 15 min at room temperature. Cells were then washed twice in PBS and once in PBS/1% BSA. Cells were permeabilized by incubation for 30 min in PBS/1% BSA supplemented with 0.1% saponin. Samples were washed with 1 ml of wash solution (1:25 dilution in water of the wash concentrate provided by the manufacturer). The samples were stained with 100 μl of 1× fluorescein isothiocyanate (FITC) conjugate (1:10 dilution of FITC conjugate with wash solution) for 60 min in the dark at room temperature. Finally, the cells were washed twice and resuspended in 1 ml of PBS for flow cytometry analysis. The amount of 8-oxiguanosine formed (Ex at 495 nm and Em at 515 nm) was measured in the MACSQuant Analyzer 10 (Miltenyi Biotec), with green fluorescence proportional to the oxidative damage caused to the DNA. Positive controls were generated after the incubation of additional samples in 800 μM Fe^{2+} and 1.7 M H_2O_2 to induce the Fenton reaction.

Simultaneous Determination of Live and Dead Spermatozoa, Caspase 3 and 7 Activity, and Lipid Peroxidation (4-HNE-Positive Cells)

This assay was a slight modification of previous protocols published by our laboratory [8, 23]. The CellEvent Caspase-3/7 Green Detection Reagent is a fluorogenic substrate for activated caspases 3 and 7. The reagent consists of a four-amino-acid peptide (DEVD) conjugated to a nucleic acid-binding dye. This cell-permeable substrate is intrinsically nonfluorescent because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of caspase 3 and caspase 7 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response with absorption/Em maxima of ~502/530 nm. Stock solutions of CellEvent (2 mM in DMSO), ethidium homodimer (1.167 mM in DMSO), and Hoechst 33342 (1.62 mM in water) were prepared. Spermatozoa (5×10^6/ml) in 1 ml of PBS were stained with 2 μl/ml of a stock solution of 0.1 mg/ml of anti-4-HNE primary antibody and incubated at room temperature in the dark for 30 min. Then, the cells were washed with PBS, followed by the addition of 2 μl/ml of secondary anti-mouse Alexa Fluor 647 antibody, 2 μM CellEvent, and 0.5 μM Hoechst 33342 and incubation for another 30 min in the dark at room temperature. Then, the cells were washed in PBS, and 0.35 μM ethidium homodimer was added to each sample. After incubation for an additional 5 min, the samples were immediately analyzed by flow cytometry. The controls consisted of unstained, single-stained, secondary antibody-only staining, and FMO controls to properly set gatings and compensations. The positive controls for 4-HNE were samples supplemented with 800 μM SO_4Fe and 1.7 M H_2O_2 (Sigma) to induce the Fenton reaction. The debris were gated out based on the DNA content of the events after H33342 staining.

Flow Cytometry Determination of Intracellular Thiol Content in Stallion Spermatozoa

Intracellular thiol content was determined using previously published protocols [7, 24]. The following stock solutions were prepared: LIVE/DEAD Fixable Far Red Dead Cell Stain kit (50 μl of DMSO in the LIVE/DEAD vial) and ThiolTracker Violet (10 mM in DMSO). The samples (1 ml) containing 6–7×10^6 spermatozoa/ml in PBS were stained with 0.5 μl of LIVE/DEAD Fixable Far Red Dead Cell Solution and 0.3 μl of ThiolTracker Violet. After thorough mixing, the samples were incubated at 38°C for 30 min in the dark. Then, the spermatozoa were washed with PBS before analysis by flow cytometry. The spermatozoa were gated out of the debris based on forward and side scatter characteristics of the events and on the positivity of live/dead staining. The positive controls were high-quality sperm obtained after single-layer centrifugation [25]. Briefly, 4 ml of a suspension of glycydopropyl-trimethoxysilane-coated silica particles (Androcol Minitub) was pipetted into a centrifuge tube. Thereafter, 1 ml of semen was layered on top of the column, and the tubes were centrifuged ($300 \times g$ for 20 min). The negative controls were samples supplemented with 800 μM SO_4Fe and 1.7 M H_2O_2 (Sigma) to induce the Fenton reaction. The spermatozoa were classified based on the intensity of the fluorescence signal (arbitrary fluorescence units) using histogram plots after gating out debris.

Statistical Analysis

The normality of the data was assessed using the Kolmogorov-Smirnov test, and the equality of the variances was assessed using the Levene test. Because the data show equivalence of variance, the results were analyzed by a paired t-test (prethaw vs. postthaw) (SPSS 19.0 for Mac). Differences were considered significant when $P < 0.05$. The results are displayed as means ± SEM. The correlations between assays with fresh samples and after cryopreservation were investigated using the Pearson correlation test. For a further definition of sperm quality, a score was given to all the sperm parameters obtained postthawing (PT), including sperm membrane integrity, motility, sperm velocity, and mitochondrial membrane potential (ΔΨm), based on descriptive statistics to establish the median, minimum, and maximum values for each sperm parameter PT. Later, PT sperm quality was classified as poor, average, or good using the 25th and 75th percentiles as thresholds, such that values equal to or below the 25th percentile were considered poor, values between the 26th and 74th percentiles were considered average, and values equal to or above the 75th percentile were considered good. Using these values, a sperm PT quality index was proposed. The sperm quality index postthaw (QIPT) is a linear combination of seven different indicators of sperm functionality PT based on previous protocols from our laboratory [26]. This index combines the evaluation of different and objectively measured sperm parameters related to membrane integrity, mitochondrial membrane potential, sperm motility, and characteristics of sperm movement PT. In this manner, we gathered data obtained from a combination of tests that may better reflect the number of sperm functions and attributes that a sperm needs to be fertile. Using the 25th and 75th percentiles as thresholds, each stallion was given an arbitrary value of 1 (poor), 2 (average), or 3 (good) for each parameter. The sum of the values of each of the parameters yielded the final QIPT. Receiver operating characteristic (ROC) curves and Youden J statistics were used to investigate the value of the proposed variables as predictors of potential freezability.

RESULTS

Identification of 8-iso-PGF$_{2\alpha}$ in Stallion Spermatozoa and Changes Induced by Cryopreservation

We identified 8-iso-PGF$_{2\alpha}$ in fresh and frozen-thawed spermatozoa using specific polyclonal antibodies and flow cytometry. Cryopreservation induced a reduction in the fluorescence of antibody-positive cells compared with fresh samples ($P < 0.01$) (Fig. 1).

Identification of 8-OH Guanosine in Stallion Spermatozoa and Changes Induced by Cryopreservation

Cryopreservation induced an important reduction in the fluorescence of the probe for oxidized DNA, determined as relative fluorescence units, from 134 in fresh samples to 37 in frozen-thawed sperm ($P < 0.01$). Low probe fluorescence was maintained after 3 h of incubation at 37°C (Fig. 2).

Identification of 4-HNE in Two Distinct Sperm Subpopulations and Changes Induced by Cryopreservation

In fresh sperm, staining for 4-HNE was more prevalent in the subpopulation of spermatozoa presenting active caspase 3 and 7. Cryopreservation increased the presence of 4-HNE in both subpopulations (live sperm and caspase 3- and 7-positive sperm); this change was more pronounced in the caspase 3-positive population of spermatozoa (Fig. 3).

Identification of Significant Correlations among Sperm Characteristics in Fresh Sperm and Sperm Quality Postthaw

Sperm characteristics in fresh samples were compared with the quality of the same samples after freezing and thawing (Table 1). Two major groups of correlations were evident. Sperm characteristics in fresh samples with high and positive correlations with favorable outcomes of cryopreservation (values of the sperm analysis included in the top 75th percentile after thawing) included spermatozoa with high mitochondrial membrane potential, live sperm negative for caspase 3, live sperm with high intracellular thiol content, and spermatozoa with higher levels of 8-iso-PGF$_{2\alpha}$ in fresh samples. Another group of parameters in raw semen was negatively correlated with sperm quality postthaw. This group included the simultaneous presence of active caspase 3 and 4-HNE in fresh samples, as well as the presence of dead sperm with active caspase 3 in fresh samples.

Of note were high correlations between the percentage of live sperm without active caspase 3 in fresh samples and the percentages of several measured parameters in frozen-thawed sperm, including motile and linear motile sperm in the same samples after freezing and thawing ($r = 0.553$, $P < 0.05$, and $r = 0.598$, $P < 0.05$), the percentages of spermatozoa with high mitochondrial membrane potential ($r = 0.795$, $P < 0.01$), live spermatozoa ($r = 0.763$, $P < 0.01$), live spermatozoa without active caspase 3 ($r = 0.780$, $P < 0.01$), and live spermatozoa with high thiol content ($r = 0.597$, $P < 0.01$). The percentage of live sperm with high thiol content in fresh samples was correlated with the postthaw percentages of motile and linear
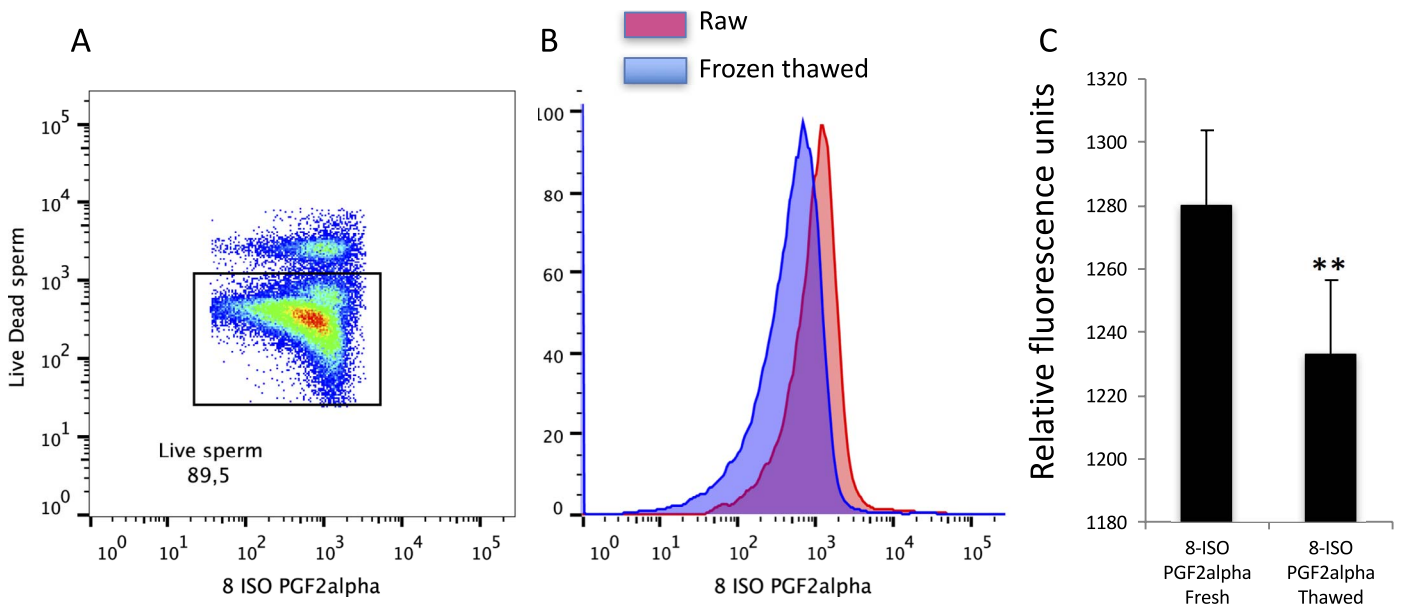
FIG. 1. Flow cytometry detection of 8-ISO-PGF$_{2\alpha}$ in stallion spermatozoa. Stallion spermatozoa were incubated with a specific primary antibody against 8-ISO-PGF$_{2\alpha}$ and a fluorescence-marked secondary antibody as described in *Materials and Methods*. A fixable live/dead probe was used to identify live and dead spermatozoa. **A**) Representative cytogram of the assay. a gate was applied to the region of live sperm cells, and this region was further analyzed for detection of the intensity of fluorescence of 8-ISO-PGF$_{2\alpha}$ antibody in raw and frozen-thawed stallion spermatozoa. **B**) Representative overlay histograms of the fluorescence intensity of the 8-ISO-PGF$_{2\alpha}$ antibody in raw and frozen-thawed stallion spermatozoa. **C**) Changes in 8-ISO-PGF$_{2\alpha}$ induced by cryopreservation. **P < 0.01.

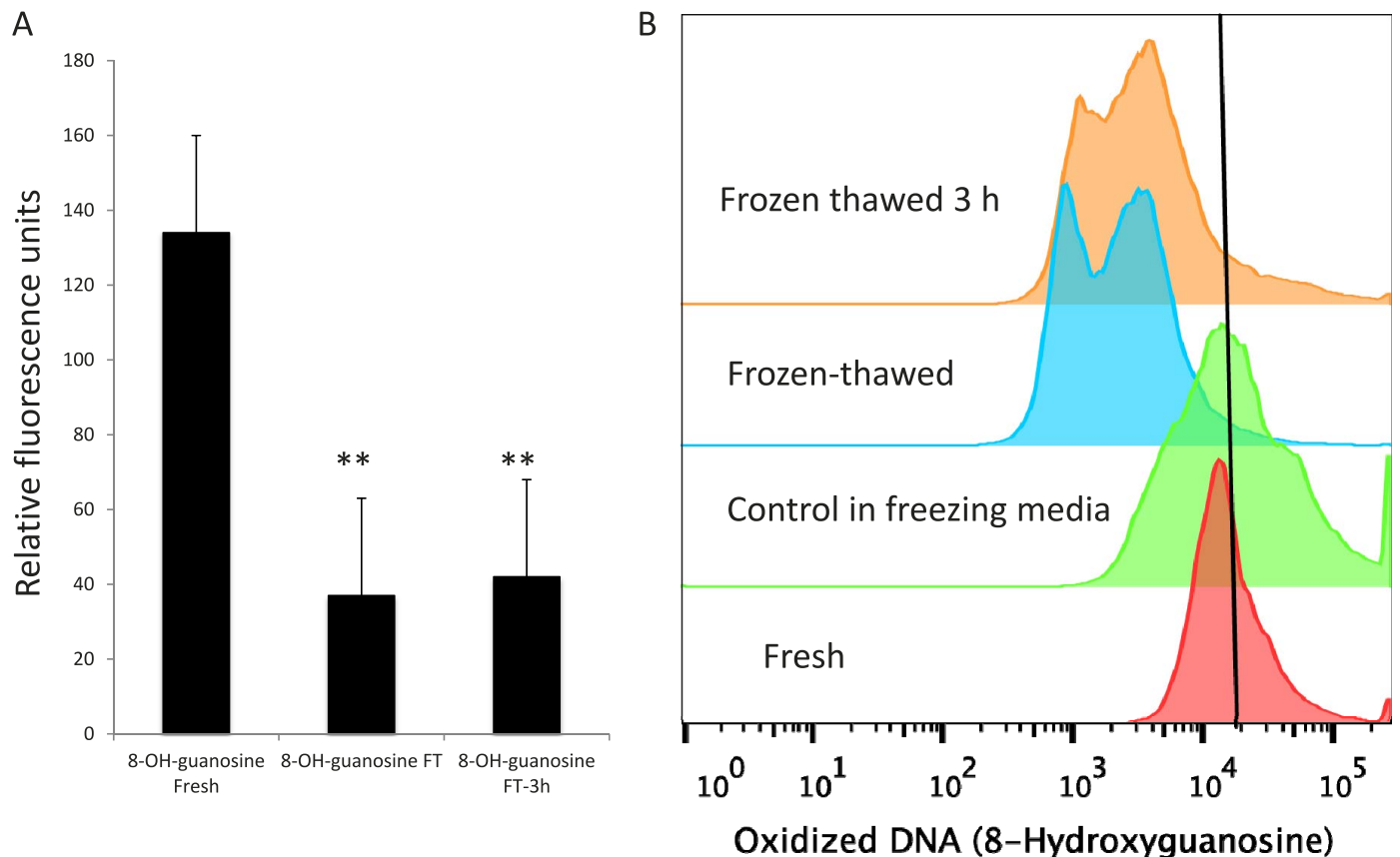**FIG. 2.** Changes in the oxidation of DNA induced by cryopreservation. DNA oxidation was investigated using a monoclonal antibody against the oxidized adduct of guanosine, 8-OH guanidine, and flow cytometry as described in *Materials and Methods*. **A**) DNA oxidation in fresh samples after thawing and after incubation of frozen-thawed samples for 180 min at 37°C; **P < 0.01. **B**) Representative cytograms.
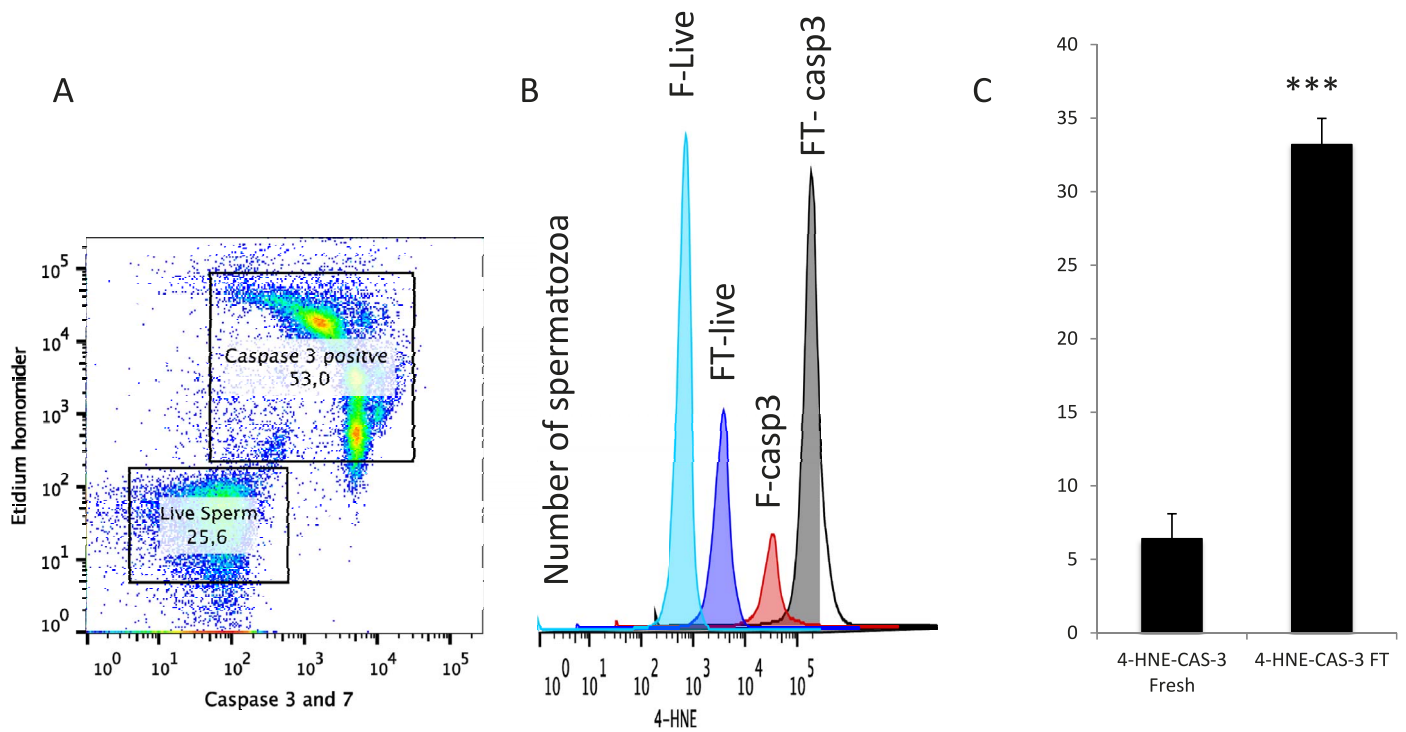
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FIG. 3.   Changes in 4-HNE during the cryopreservation process and identification of the sperm subpopulation with a higher 4-HNE. **A**) Identification of caspase 3-positive and -negative spermatozoa. **B**) Identification of 4-HNE-positive spermatozoa in the populations of live and caspase 3-positive spermatozoa and changes induced by cryopreservation; 4-HNE was present mainly in the population of caspase 3-positive sperm and increased in both populations after cryopreservation. **C**) Changes in the presence of 4-HNE induced by cryopreservation; ***$P < 0.01$.

motile sperm ($r = 0.628$, $P < 0.05$, and $r = 0.659$, $P < 0.05$), spermatozoa with high mitochondrial membrane potential ($r = 0717$, $P < 0.01$), live spermatozoa without active caspase 3 ($r = 0.650$, $P < 0.01$), and live sperm ($r = 0.571$, $P < 0.05$).

Interestingly, the presence of 8-iso-PGF$_{2\alpha}$ in fresh samples was correlated with the percentage of live sperm with high thiol content in the frozen-thawed samples ($r = 0.552$, $P < 0.05$). It is noteworthy that negative correlations existed between the

TABLE 1.   Correlations between oxidative apoptotic and mitochondrial status in fresh samples and values of sperm analysis in the same samples after freezing and thawing.

Assays performed in raw semen	Assays performed in the same samples after freezing and thawing	Correlation
Caspase 3- and 4-HNE-positive sperm	High mitochondrial membrane potential	$r = -0.520*$
	Live caspase-negative sperm	$r = -0.616*$
	Live sperm with high thiol content	$r = -0.568*$
High mitochondrial membrane potential	Motile sperm	$r = 0.555*$
	Linear motile spermatozoa	$r = 0.560*$
	High mitochondrial membrane potential	$r = 0.717**$
8-iso-PGF$_{2\alpha}$	Live sperm with high thiol content	$r = 0.552*$
Live caspase 3-negative sperm	Motile sperm	$r = 0.553*$
	Linear motile sperm	$r = 0.598*$
	High mitochondrial membrane potential	$r = 0.795**$
	Live caspase 3-negative sperm	$r = 0.780**$
	Live sperm	$r = 0.763**$
	Live sperm with high thiol content	$r = 0.597*$
Caspase 3 dead sperm	Motile spermatozoa	$r = -0.549*$
	High mitochondrial membrane potential	$r = -0.729**$
	Live caspase 3-negative spermatozoa	$r = -0.814**$
	Live spermatozoa	$r = -0.820**$
	Live spermatozoa with high thiol content	$r = -0631**$
Live spermatozoa with high thiol content	Motile spermatozoa	$r = 0.628*$
	Linear motile spermatozoa	$r = 0.659*$
	High mitochondrial membrane potential	$r = 0.717**$
	Live caspase 3-negative spermatozoa	$r = 0.650**$
	Live spermatozoa	$r = 0.571*$

* $P < 0.05$.
** $P < 0.01$.

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TABLE 2. Descriptive statistics for selected parameters of sperm quality postthaw used to classify stallion ejaculates as good or bad freezers.

Parameter[a]	Mean ± SD	Minimum	Maximum	25th percentile	50th percentile	75th percentile
Motile %	47.9 ± 13.6	26	79	38.5	47.5	55.5
Linear motile %	27.6 ± 9.3	13	46	20.0	30.0	34.2
VCL μm/sec	116.6 ± 16.1	16.1	145	100.7	117.0	129.7
VSL μm/sec	35.6 ± 6.8	25	47	30.7	33.5	42.5
VAP μm/sec	58.6 ± 7.6	46	72	51.7	59	64
Live Casp 3- sperm %	37.5 ± 9.1	23.1	49.5	27.9	41.1	45.1
Live sperm	40.4 ± 8.8	24.8	52.3	31.6	43.9	47.6
Live sperm with high thiol content	35.1 ± 10.1	17.1	48.7	26.3	39.0	42.7
Live sperm with high ΔΨm	38.2 ± 9.1	24.1	50.1	28.4	41.6	46.0

[a] VCL, circular velocity; VSL, straight line velocity; VAP, average velocity; Casp 3-, caspase 3 negative.

percentages of dead sperm with active caspase 3 in fresh samples ($r = -0.820$, $P < 0.01$) and the percentage of spermatozoa with high mitochondrial membrane potential in frozen-thawed samples ($r = -0.729$, $P < 0.01$).

Sperm Quality Postthaw

The values of the QIPT are given in Table 2. Significant differences were observed between stallions in PT sperm quality (Fig. 4A), and these differences were not always coincident with the traditional estimates of PT sperm quality of motility and linear motility (Fig. 4, B and C). To further

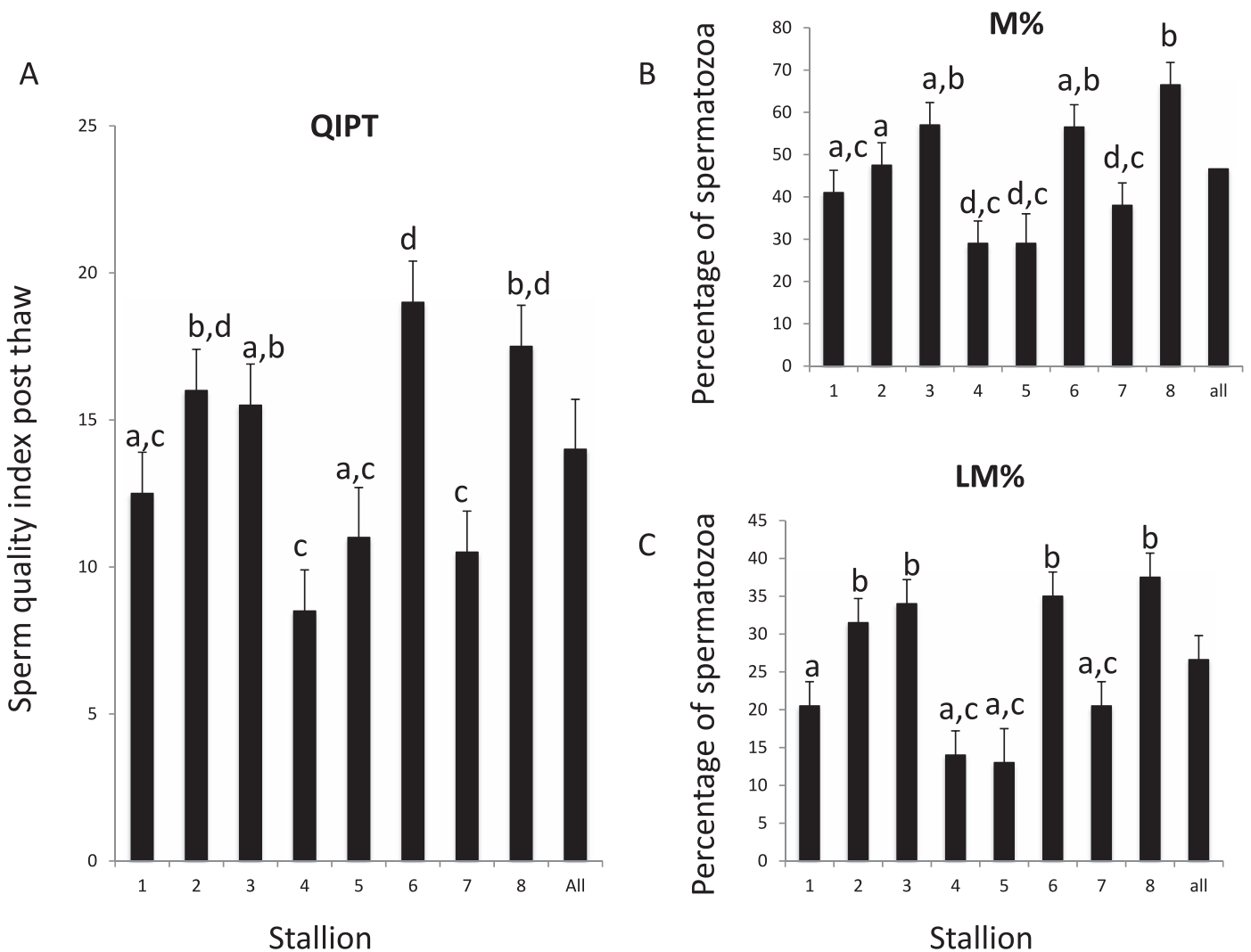FIG. 4. Outcome of cryopreservation in eight stallions. **A**) Sperm quality index. This is a linear combination of nine characteristics of sperm quality as described in *Materials and Methods* and in Table 2. **B** and **C**) Outcome of cryopreservation in terms of percentages of motility and linear motility postthaw. Values with different superscripts a, b, c differ significantly ($P < 0.05$).

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Cat Motile

A

Cat Lin Motile

B

Cat VCL

C

Cat VAP
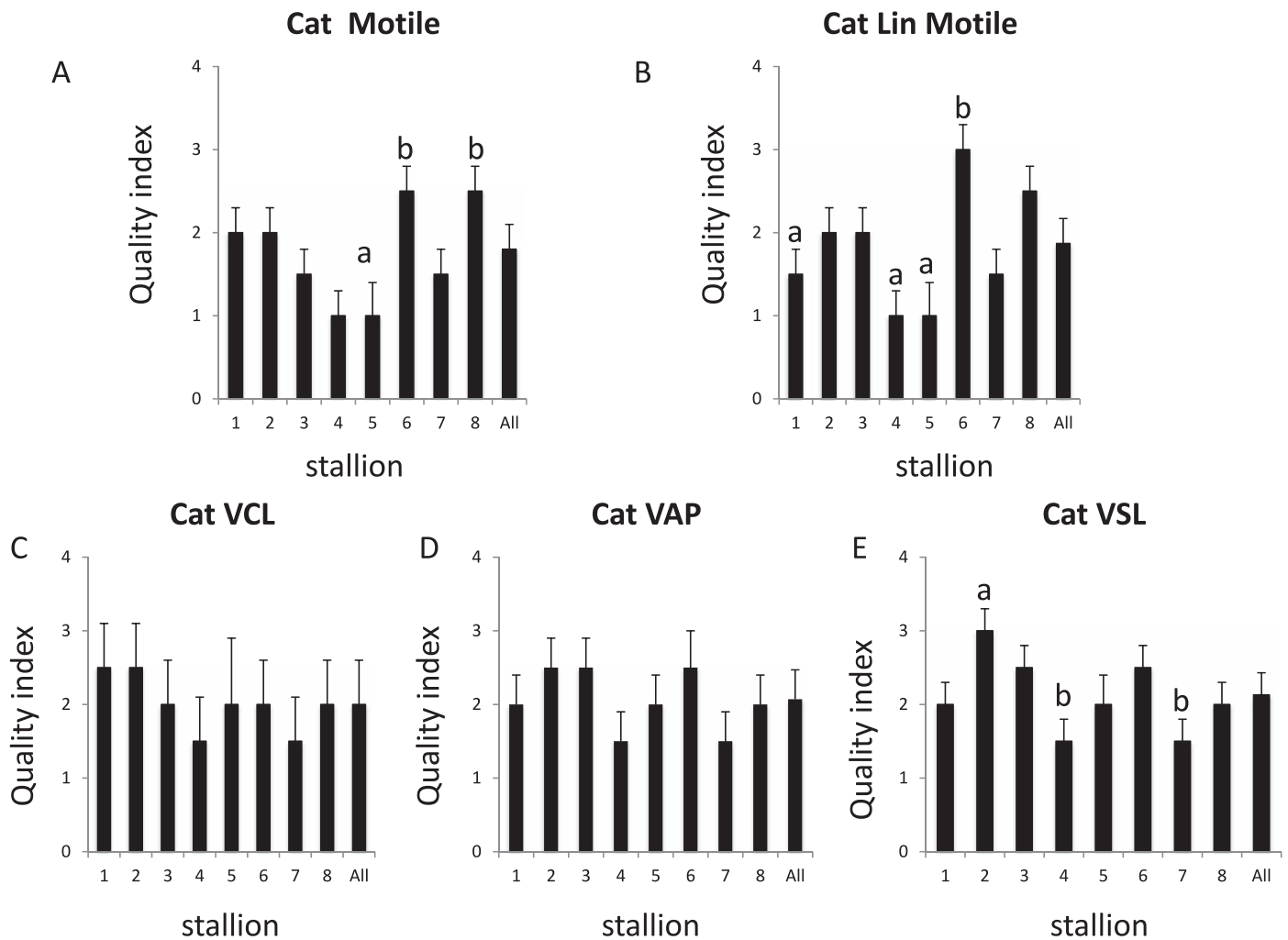
D

Cat VSL

E

FIG. 5. Specific sperm quality indexes postthaw in eight stallions. Ejaculates were classified as described in *Materials and Methods* to determine good and bad freezers according to the specific sperm quality indexes developed using the thresholds described in Table 2. **A**) Motility quality index. **B**) Linear motile quality index. **C**) Circular velocity quality index. **D**) Average velocity quality index. **E**) Straight-line velocity quality index. Values with different superscripts a, b, c, d differ significantly ($P < 0.05$).

disclose the factors involved in the proposed global index, specific partial indexes were also proposed (Figs. 5 and 6).

Prediction of the Outcome of Cryopreservation Using ROC Curves

The parameters of fresh sperm that were significantly correlated with sperm functionality postthaw were further investigated using ROC curves and Youden J statistics. We identified several sperm characteristics in fresh sperm with potentially high predictive values (Table 1) based on correlations. The ROC curves revealed several sperm parameters of fresh sperm that predict sperm quality postthaw, namely, either bad freezers (Fig. 7) or good freezers (Figs. 8 and 9), both referring to particular sperm parameters postthaw (Fig. 8), or combined indexes of sperm quality (Fig. 9). Interestingly, predictive parameters of sperm quality were related to reduced levels of active caspase 3 and 7, intense mitochondrial activity, and high antioxidant defense mechanisms in fresh sperm, although paradoxical findings were also evident, as a marker of oxidative stress (8-iso PGF2α)

predicted the outcome of cryopreservation with respect to the amount of live sperm postthaw (Fig. 8C).

DISCUSSION

In this study, we investigated the presence of and cryopreservation-induced changes in specific markers of oxidative stress, as well as the potential of markers of sperm senescence to forecast the outcome of cryopreservation. Three markers of oxidative stress were investigated, namely DNA oxidation (8-OH-guanosine) and two markers of lipid peroxidation, 8-iso $PGF_{2\alpha}$ and 4-HNE. For the first time, we identified in stallion spermatozoa the presence of 8-iso $PGF_{2\alpha}$, and paradoxical results were obtained. Although 8-OH-guanosine and 8-iso $PGF_{2\alpha}$ levels decreased in cryopreserved samples, 4-HNE increased after thawing. These paradoxical results can be explained based on recent findings on the unique biology of stallion spermatozoa, whereby oxidative phosphorylation is particularly active [9, 11]. In stallions, more fertile spermatozoa also produce more ROS, particularly the superoxide anion ($O_2^{\bullet-}$). This finding may explain the reduced DNA oxidation and lower 8-iso

A
B
C

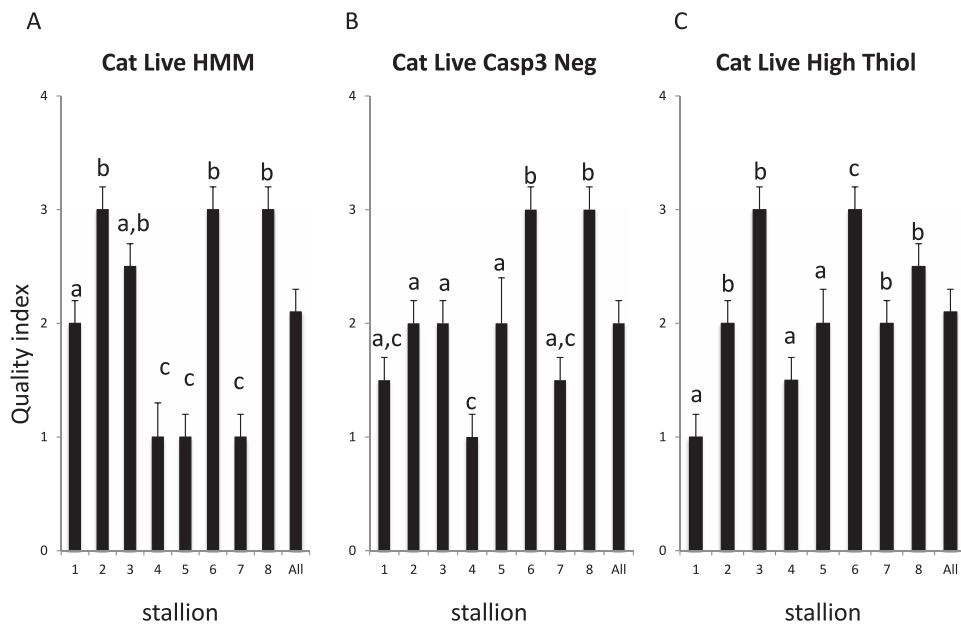**Cat Live HMM**   **Cat Live Casp3 Neg**   **Cat Live High Thiol**

FIG. 6.   Specific sperm quality indexes postthaw in eight stallions. Ejaculates were classified as described in *Materials and Methods* to determine good and bad freezers according to the specific sperm quality indexes developed using the thresholds described in Table 2. **A**) Sperm quality index refers to the percentage of live sperm with high mitochondrial membrane potential postthaw. **B**) Sperm quality index refers to the percentage of live sperm negative for caspase 3. **C**) Sperm quality index refers to the percentage of live sperm with high thiol content postthaw. Values with different superscripts differ significantly.

$PGF_{2\alpha}$ levels observed in the cryopreserved samples, as it is known that cryopreservation impairs mitochondrial function [10, 15, 27]. If mitochondrial function is reduced, ROS production and, therefore, DNA oxidation and 8-iso $PGF_{2\alpha}$ are also reduced. The positive correlations observed between 8-iso $PGF_{2\alpha}$ levels in fresh samples and live sperm postthaw also support this hypothesis, as does the fact that the 8-iso $PGF_{2\alpha}$ levels were higher in live sperm, as the increased production of ROS reflects increased mitochondrial activity in stallion spermatozoa [9]. Interestingly, 8-iso $PGF_{2\alpha}$ correlated with cryoresistance, providing further evidence supporting this hypothesis, as recent reports have linked good freezability with higher levels of ROS in stallions [10]. In contrast, 4-HNE increased after cryopreservation, particularly in the population of spermatozoa that also showed caspase activity, confirming previous findings [7]. These different effects may relate to the origin of both compounds. 8-iso-$PGF_{2\alpha}$ is a member of the isoprostane class of prostanoids, which are formed by free radical-mediated oxidation of arachidonic acid, whereas 4-HNE arises from the oxidation of ω-6 fatty acids. It is thought that 4-HNE, although triggered by an initial oxidative step, can later be decoupled from oxidative stress and its production continues if a source of ω-6 fatty acids [28] is present. Stallion spermatozoa are rich in ω-6 fatty acids, particularly docosapentanoic acid (C22:5n-6), representing on average half of all the fatty acids, whereas arachidonic acid is present at much lower levels [18]. Moreover, the toxicity of 4-HNE to stallion spermatozoa has been well documented [9, 29], whereas the toxic effects of 8-iso-$PGF_{2\alpha}$ and 8-OH-guanosine on spermatozoa are unknown, probably representing only markers of ROS production, and their generation depends on a continuous source of ROS. However, is important to note that frozen-thawed sperm, in contrast to raw sperm, have been in contact with egg yolk,

which is an antioxidant, and when inseminated, the sperm readily swim out of this protection and are likely more susceptible to oxidative stress in the mare's reproductive tract.

We also investigated which parameters in raw semen could predict the outcome of cryopreservation. We revealed parameters able to predict both good and bad outcomes of this procedure. To determine which ejaculates performed the best after cryopreservation, we considered as good freezers those falling in the best 75th percentile and bad freezers as those falling in the 25th percentile. We also developed combined indexes of sperm quality postthaw following these criteria using previous protocols from our laboratory [26]. The percentage of caspase 3-positive dead sperm was highly correlated with the worst outcome of cryopreservation and, based on ROC curves and the Youden index, was able to detect ejaculates that would not freeze well. Specifically, using percentiles to classify ejaculates, we were able to predict ejaculates falling in the 25% worst values of sperm quality postthaw. This result can be easily explained based on recent developments in elucidating the meaning of spermptosis [8] in the ejaculate. Caspase 3-positive spermatozoa are immature sperm appearing in the ejaculate [30]. It has recently been demonstrated that dead sperm in the ejaculate are detrimental to the live sperm [14], especially in the presence of egg yolk, a common component of cryopreservation media. Egg yolk acts a source of aromatic amino acids as substrate for a L-amino acid oxidase present in stallion sperm [14]. These two facts explain the predictive value of caspase 3-positive dead sperm in the ejaculate in detecting ejaculates that will show poor results after cryopreservation, confirming previous reports indicating that apoptotic markers may predict freezability [31–33]. Cryopreservation is an inducer of apoptotic changes in somatic and germ cells [27, 31, 34–37]; thus, it is not

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A) AUC= 0.864, p<0.01
Cut off value after Youden´s index
9.50 C3-4-HNE
Live sperm caspase 3 negative PT

B) AUC= 0.972, P<0.05
Cut off value of Youden's Index
8.44 C3-4-HNE
Live sperm with high thiol content PT

C) AUC= 0.820, P<0.05
Cut off value after Youden's index 7,58
Casp 3 and Eth +
Motile Sperm PT

D) AUC= 1,0, P<0.01
Cut off value after Youden's index 7,58
Casp 3 and Eth+
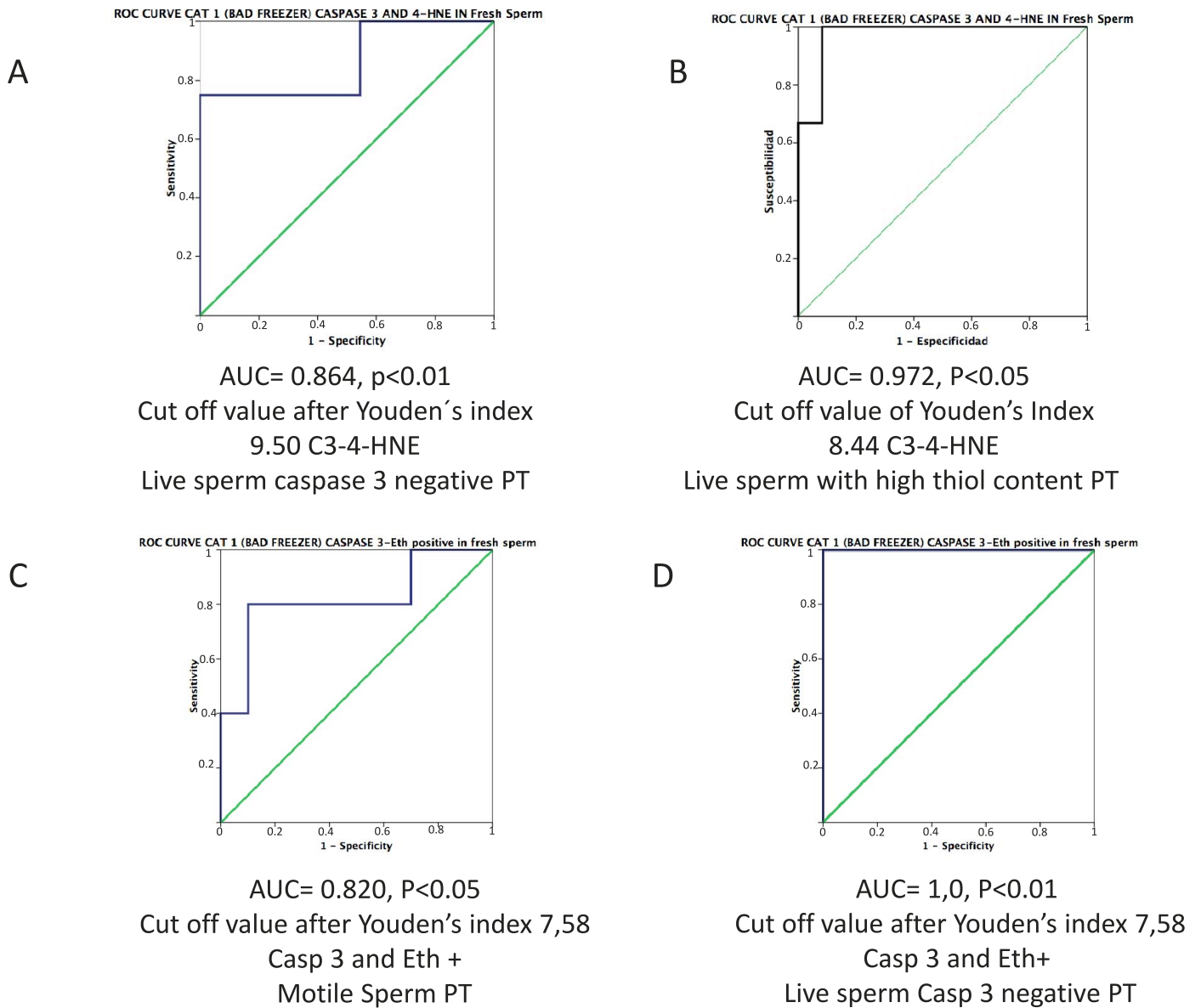Live sperm Casp 3 negative PT

FIG. 7. ROC curves for the detection of bad freezers. **A**) The percentage of caspase 3- and 4-HNE-positive sperm in fresh samples predicted the outcome of cryopreservation in terms of the percentage of live spermatozoa and caspase 3-negative spermatozoa postthaw. When the percentage of caspase 3- and 4-HNE-positive sperm was higher than 9.5% in fresh samples, the ejaculate did not freeze well. **B**) Caspase 3-positive 4-HNE live sperm with a thiol content above 8.44% predicted poor freezability in fresh samples. **C**) Forecast of the outcome of cryopreservation in terms of total motile sperm postthaw when the percentage of dead sperm with active caspase 3 in fresh samples was over 7.58%. **D**) Forecast of the outcome of cryopreservation in terms of live caspase 3-negative sperm postthaw when the percentage of dead sperm with active caspase 3 in fresh samples was over 7.6%.

surprising that if basal spermptotic status is high before cryopreservation, higher sperm mortality will result, especially taking into account the fact that the presence of dead cells also activates cellular damage [14, 38]. In addition, the presence of caspase 3- and 4-HNE-positive sperm identified ejaculates with poor freezability, and the explanation of this finding is very similar to that for the previous sperm category. Lipid peroxidation, and 4-HNE in particular, is a potent inducer of spermptosis in stallion spermatozoa [7], and during cryopreservation, ejaculates with higher amounts of 4-HNE experience caspase activation and poorer cryosurvival.

We were also able to predict positive outcomes of cryopreservation. In general terms, the quality of fresh samples expressed as the absence of spermptotic features predicted the best outcome of cryopreservation. This finding can be easily explained in view of recent findings linking the inhibition of spermptosis [8] and sperm survival after ejaculation [19, 21, 39] and the fact that cryopreservation accelerates this particular form of apoptosis in sperm [4, 23, 31]. It is possible that the less activated the spermptotic process is in fresh sperm, the less progression there is to death and senescence induced by the freezing procedure. An interesting and apparently paradoxical finding was the relationship between 8-iso-PGF$_{2\alpha}$ and the percentage of live sperm after cryopreservation. However, similar findings have been recently reported [9] and are explained by the great dependence of stallion spermatozoa on oxidative phosphor-
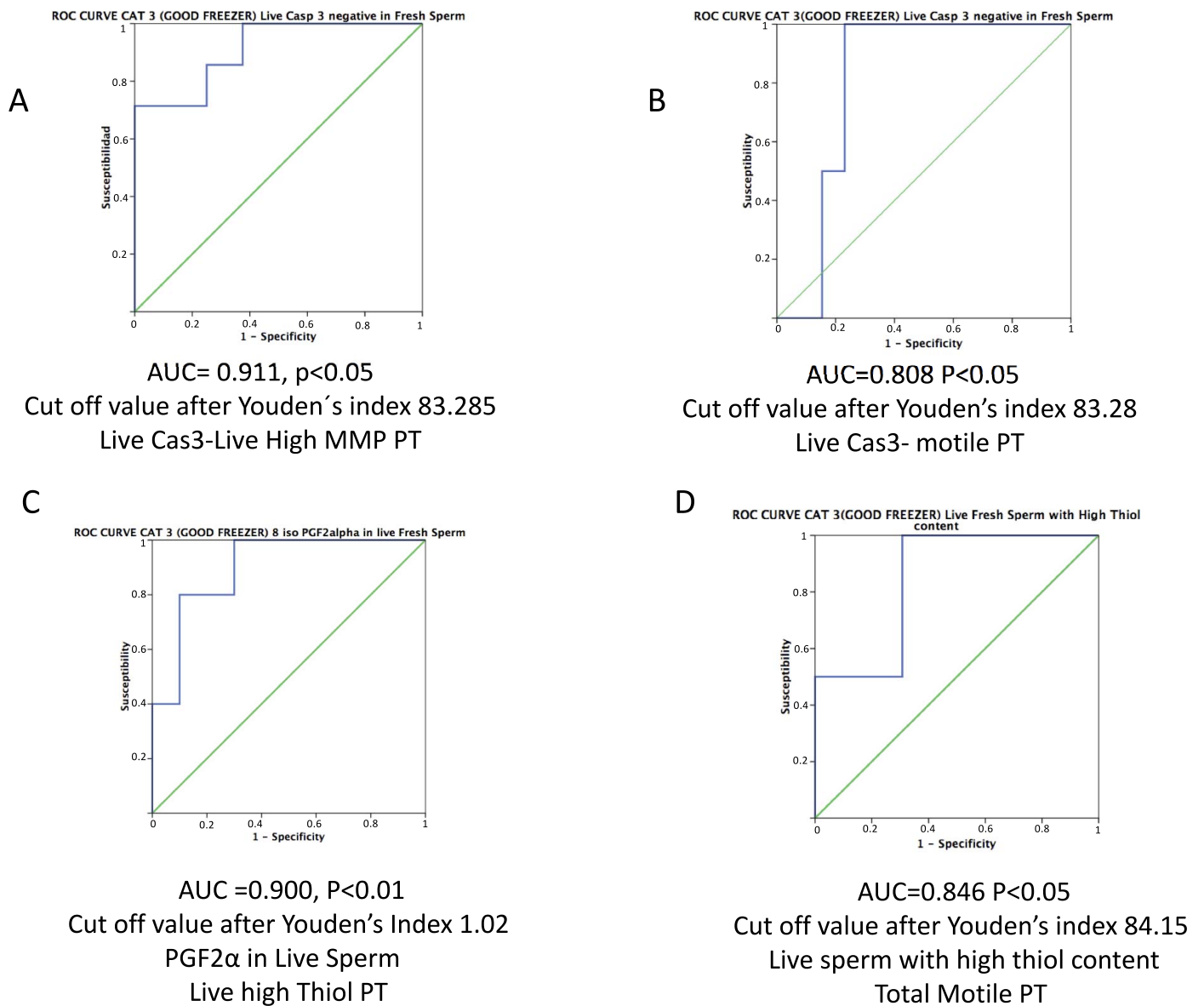
A

AUC= 0.911, p<0.05
Cut off value after Youden's index 83.285
Live Cas3-Live High MMP PT

B

AUC=0.808 P<0.05
Cut off value after Youden's index 83.28
Live Cas3- motile PT

C

AUC =0.900, P<0.01
Cut off value after Youden's Index 1.02
PGF2α in Live Sperm
Live high Thiol PT

D

AUC=0.846 P<0.05
Cut off value after Youden's index 84.15
Live sperm with high thiol content
Total Motile PT

FIG. 8.   ROC curves for the detection of good freezers. The percentage of live caspase 3-negative sperm predicted the outcome of cryopreservation (**A**) in terms of live sperm with high mitochondrial membrane potential when the percentage of live caspase 3-negative sperm was over 83.3%, (**B**) in terms of total motile sperm postthaw when the percentage of live caspase 3-negative sperm was over 83.3%, (**C**) in terms of live sperm with high thiol content postthaw when the percentage of spermatozoa positive for 8-iso PFG2α was over 1.02%, and (**D**) in terms of total motile sperm postthaw when the percentage of live sperm with high thiol content in fresh samples was 84.1%.

ylation in the mitochondria as the main source of ATP [9, 11, 40]. Intense mitochondrial activity results in increased ROS production, particularly $O_2^{\bullet -}$. Moreover, a previous study reported higher levels of ROS in frozen-thawed sperm, with better freezability linked to mitochondrial activity [10, 41]. More robust spermatozoa have intense mitochondrial activity. Another subpopulation of spermatozoa with good cryopreservation outcomes was live sperm with high thiol content. Thiols, particularly reduced glutathione, are pivotal mechanisms for the maintenance of ROS homeostasis in spermatozoa [42, 43]. Because cryopreservation is a major disruptor of ROS homeostasis [5, 41], ejaculates with high thiol content survive the procedure better. Recently, a similar approach to predict field fertility of frozen-thawed sperm has been reported [44], further stressing the importance of multi-

parametric sperm analysis and the importance of mitochondrial assessment.

In summary, we have determined the differential effects of ROS during cryopreservation, namely, decreased DNA oxidation, reduced levels of 8-iso-$PGF_{2\alpha}$, and increased production of 4-HNE. In addition, we demonstrated that good outcomes of cryopreservation are linked to reduced caspase 3 activity and high thiol content in fresh samples. These findings provide new clues to the understanding of the importance of ROS homeostasis in stallion spermatozoa and may have rapid practical application in the andrological evaluation of stallions and in the selection of ejaculates for freezing.
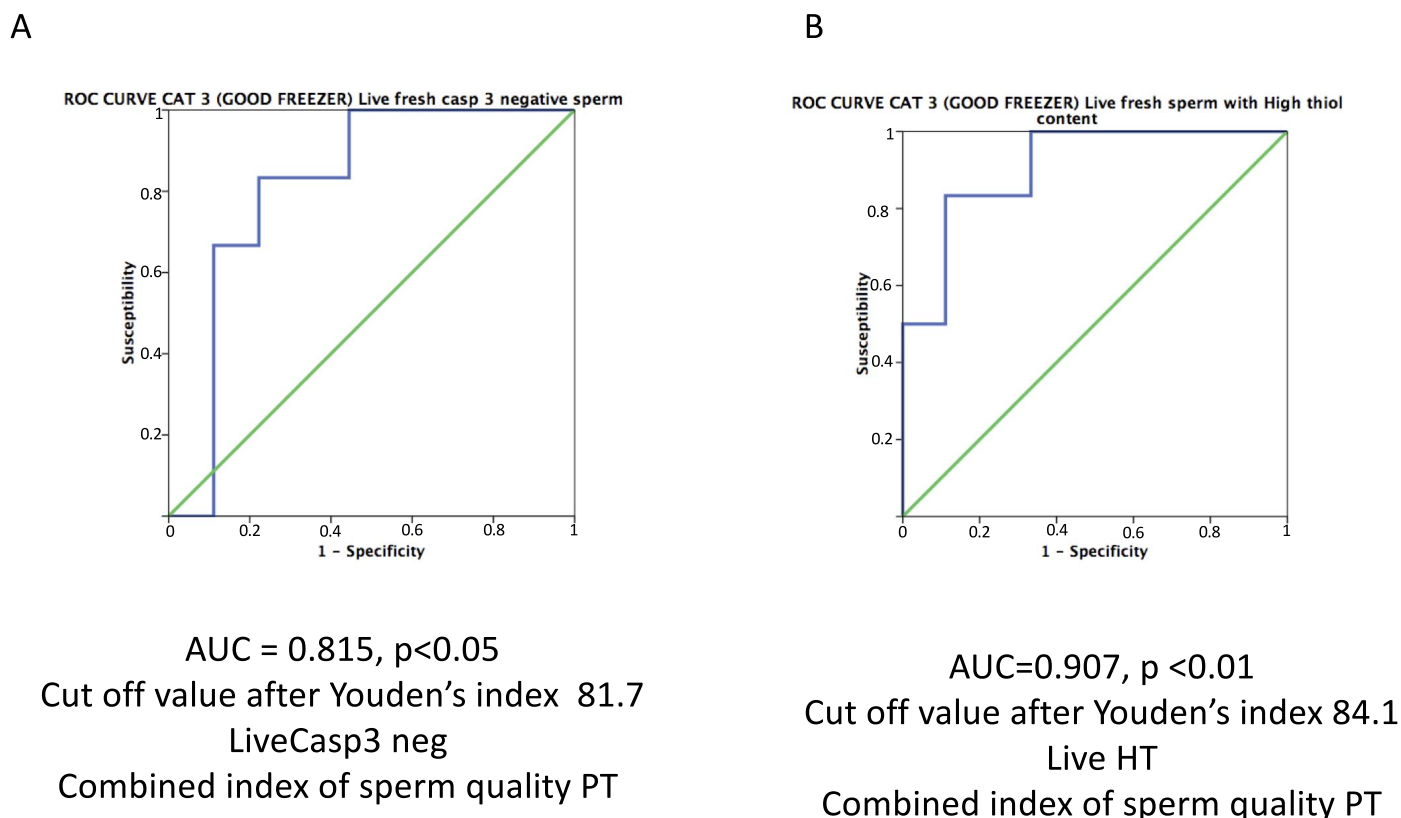
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A

AUC = 0.815, p<0.05
Cut off value after Youden's index 81.7
LiveCasp3 neg
Combined index of sperm quality PT

B

AUC=0.907, p <0.01
Cut off value after Youden's index 84.1
Live HT
Combined index of sperm quality PT

FIG. 9.   ROC curves for the detection of good freezers in terms of the combined index of sperm quality postthaw in (**A**) live caspase 3-positive sperm in fresh samples when the percentage of spermatozoa falling within this category was over 81.7% and (**B**) live sperm with high thiol content in fresh samples when the percentage of spermatozoa in this category was over 84.1%.

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